# FRACTIONATION OF NEUTRAL AND SIALYLATED N-LINKED GLYCANS USING MICRO SCALE HILIC SPE FOR MALDI-TOF MS ANALYSIS

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## OVERVIEW

- A micro scale hydrophilic interaction chromatography (HILIC) SPE method has been developed to fractionate and enrich N-linked glycans released from glycoproteins.
- Low and high pH stepped fractionations enable to isolate the sialylated glycans, therefore, simplifying the identification and structural characterization of these glycans using MALDI MS analysis.

## INTRODUCTION

Characterization of oligosaccharides from glycoproteins is challenging due to their structural heterogeneity and low abundance. Glycans released from glycoproteins contain both neutral and acidic glycans. Extensive fragmentation of sialylated glycans (loss of sialic acids) occurs with MALDI and multiple peaks are generally observed as a result of the fragmentation. Since the desialylated fragment ions can have the same mass as the corresponding native neutral glycans, this complicates glycan assignment.
HILIC SPE was used for the removal of salts and detergents from hydrophilic analytes such as oligosaccharides. In addition, we developed a method for SPE fractionation of glycans based on their acidity. The fractionation is performed using two elution steps (Scheme 1). The first elution uses an acidic solution and neutral glycans are released from the SPE exclusively; the second elution is a pH neutral solution which elutes the remaining glycans that contain acidic glycans only. The fractionated glycans are analyzed separately, which reduces the complexity of the sample, allows an easier assignment of sialylated glycans.

## METHODS

### Deglycosylation

Glycoproteins, RNase B, and α1-acid glycoprotein (Sigma) were solubilized in 0.1% surfactant, RapiGest™ SF (Waters). DTT was added to the solution to a final concentration of 10mM before heating the sample at 100ºC for 5 minutes. The protein sample was buffered using equal volume of 100 mM $NH_4HCO_3$. PNGase F (Sigma) was used to remove N-linked glycans.

The released glycans were desalted using Waters 96 well MassPREP™ HILIC μElution plate.
Sialic acids from AGP glycans were removed by using neuraminidase (sigma).

*Waters MassPREP™ Glyco analysis Kit contains a 96 well HILIC μElution Plate, RapiGest SP™, and DHB.*




*Scheme 1. A 96 well micro scale HILIC SPE plate operated via a vacuum manifold was used to extract and fractionate neutral and acidic glycans.*

### MALDI MS

- Waters MALDI micro MX™ , MALDI QTof Ultima™
- MALDI Matrix: MassPREP™ DHB (20 mg/ml in 100% Ethanol)
- HILIC SPE extracted sample was mixed with MALDI matrix in 1:1 ratio directly on the target and dried at ambient temperature. The glycans spotted on the MALDI target were released from 10 to 100 pmol of proteins
- MS/MS fragmentation was performed on the AGP glycans after neuraminidase treatment

## RESULTS

### 1. High pH elution buffer elutes acidic Glycan (A2F)

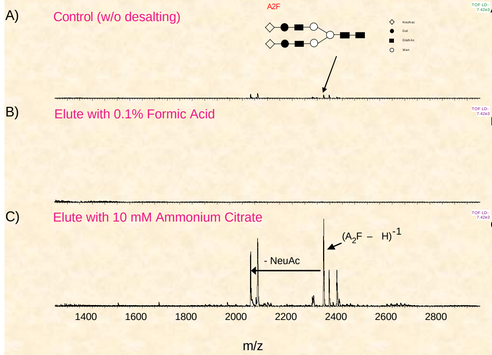



*Figure 1. MALDI-TOF MS negative ion analysis of 25 ng of sialylated glycan A2F. A) A2F Control sample analyzed directly without desalting. B) Low pH elution contained no A2F in 0.1% formic acid. HILIC μElution SPE was used to desalt A2F. C) High pH elution contained A2F in 10 mM ammonium citrate. The acidic glycan was well enriched.*

### 2. Fractionation of neutral and acidic glycan

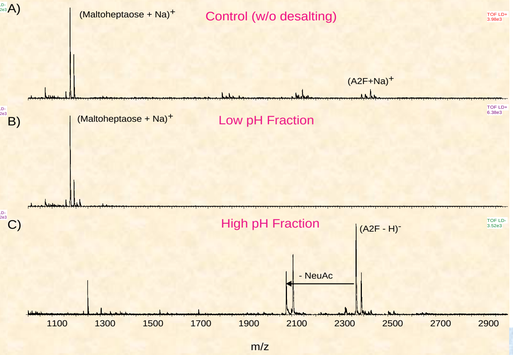
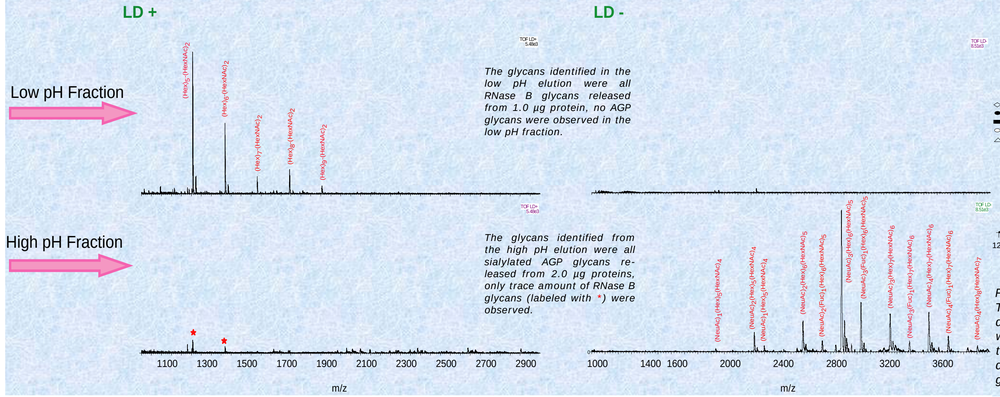



*Figure 2. MALDI-TOF MS of mixed glycans. A) Control of mixed neutral glycan (Maltoheptaose) and acidic glycan (A2F) on MALDI TOF MS positive ion mode. Extensive fragmentation of A2F ions were observed. B) Low pH elution on MALDI-TOF positive ion mode shown that Maltoheptaose was isolated in the first fractionation. C) High pH elution on MALDI-TOF negative ion mode shown that the A2F was isolated in the second elution.*

### 3. Mixed glycans released from glycoprotein, RNase B and α1-Acid Glycoprotein (AGP)

MALDI-TOF MS in both positive and negative ion modes of mixed glycans. RNase B contains neutral glycans while AGP contains only sialylated glycans.




### 4. MS/MS structure characterization of AGP glycans after the neuraminidase treatment

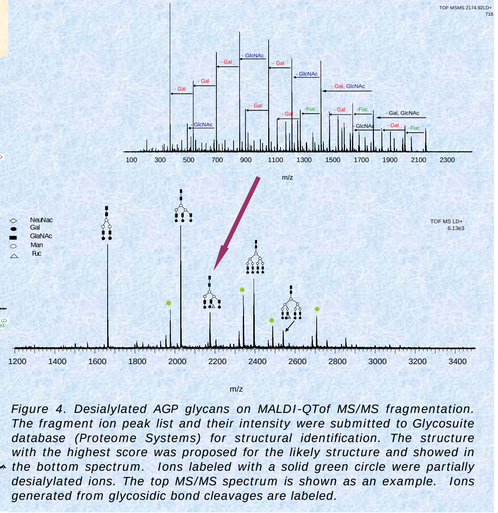



*Figure 4. Desialylated AGP glycans on MALDI-QTof MS/MS fragmentation. The fragment ion peak list and their intensity were submitted to Glycosuite database (Proteome Systems) for structural identification. The structure with the highest score was proposed for the likely structure and showed in the bottom spectrum. Ions labeled with a solid green circle were partially desialylated ions. The top MS/MS spectrum is shown as an example. Ions generated from glycosidic bond cleavages are labeled.*

## CONCLUSIONS

- HILIC μElution Plate in Waters MassPREP Glycoanalysis Kit removes salts and detergents from glycans released from glycoproteins enzymatically prior to mass spectrometry analysis
- The low and high pH stepped elutions can effectively fractionate neutral and acidic glycans (sialylated), since
  1. Neutral glycans retain on HILIC SPE due to hydrophilic interaction
  2. Acidic glycans retain on HILIC SPE due to both hydrophilic interaction and weak anion exchange mechanism
- The fractionation of glycans simplifies glycan samples and the sialylated glycan assignment

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